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1. **Your reference** — 8.68798/002.hd

2. **Patent application number** *(The Patent Office will fill in this part)* — **9909962.4**

E443880-1 D00027
0 0.00 - 9909962.4

3. **Full name, address and postcode of the or of each applicant** *(underline all surnames)*

St. George's Enterprises Ltd.
Cranmer Terrace
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London SW17 0RE

Patents ADP number *(if you know it)* — 7376957001

If the applicant is a corporate body, give country/state of incorporation — GB

4. **Title of the invention** — Improvements in or relating to screening antibacterial agents

5. **Name of your agent** *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

179 Queen Victoria Street
London
EC4V 4EL

Patents ADP number *(if you know it)* — 166001

6. **If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and** *(if you know it)* **the or each application number**

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. **If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application**

Number of earlier application	Date of filing *(day / month / year)*

8. **Is a statement of inventorship and of right to grant of a patent required in support of this request?** *(Answer 'Yes' if:*
   a) any applicant named in part 3 is not an inventor, or
   b) there is an inventor who is not named as an applicant, or
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Continuation sheets of this form	-
Description	13
Claim(s)	2
Abstract	-
Drawing(s)	3 + 3

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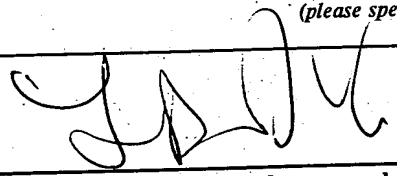
Priority documents	-
Translations of priority documents	-
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature  Date 29 April 1999

12. Name and daytime telephone number of person to contact in the United Kingdom

John Christopher Marsden
0171 206 0600

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68798/002.604

Improvements in or relating to screening antibacterial agents

The present invention relates to the isolation of subpopulations of stationary phase bacteria which exhibit resistance to conventional antibacterial agents, to the use of such resistant subpopulations in screening processes for the identification of new and improved antibacterial agents, to novel antibacterial agents identified thereby, and to therapeutic applications of such antibacterials.

Tuberculosis remains a serious disease throughout the world and it has been estimated that deaths resulting from tuberculosis account for 7% of the total number of deaths from infectious diseases. As discussed in US Patent No. 5,700,925, the vast majority of individuals who become infected with *Mycobacterium tuberculosis* do not develop symptomatic tuberculosis but do exhibit a positive reaction to the tuberculin skin test. In such infected hosts, some bacteria persist in a dormant or latent state in which they are substantially resistant to antimicrobial drugs. Over a lifetime, up to 10% of these infected but asymptomatic individuals may go on to develop tuberculosis, often many years after primary infection, when the dormant bacilli become activated and start to grow. This can result in pulmonary tuberculosis and other variant forms of the disease. Factors which predispose towards activation of the dormant organism and manifestation of the diseased state include poverty, poor living conditions, malnutrition, immune deficiency or immune suppression.

In general in the treatment of tuberculosis, antimicrobial therapy using antibacterial agents such as rifampicin, isoniazid and/or pyrazinamide is relatively

successful against actively growing bacteria. Such therapy is ineffective, however, against bacteria which remain dormant or which, having undergone a growing phase, re-enter a dormant phase. This causes particular problems in the clinical treatment of tuberculosis sufferers and carriers because the resistance of the dormant organisms necessitates long term chemotherapeutic care. Such long term treatment, typically of six months duration, is unsatisfactory since it is expensive, may result in poor patient compliance and may encourage the development and emergence of antibiotic resistant strains of bacteria over and above the *M. tuberculosis* targetted during therapy.

It is believed that most pathogenic bacteria for example *Staphyloccus aureus*, *Haemophilus influenzae*, *Streptoccus pyogenes*, *Streptococcus gordonii* and *E. coli*, possess a similar substantially antibacterial agent-resistant subpopulation which may act as a pool for reinfection during or after chemotherapy. These persistent bacteria are usually drug-sensitive at relapse, indicating that their resistance to chemotherapy is phenotypic rather than genetic.

In order to investigate whether the metabolism of such persistant bacteria is switched off with no cell division (i.e. is spore-like) or is active (i.e. so that the cells will contain markers of metabolism such as mRNA), we have studied *M. tuberculosis* in an *in vitro* stationary phase model obtained by long term culturing of the organisms in a microaerophilic gradient in which the stationary phase organisms are viable. Such stationary phase bacteria were found to be resistant to rifampicin at the normal minimum inhibitory concentration (MIC) level of 0.1 μg/ml.

The present invention is based on the unexpected and surprising finding that, whilst treatment of such stationary phase bacteria with antibacterial agents such

as rifampicin at concentrations greatly exceeding the minimum inhibitory concentration reduces plate counts to zero colony forming units (CFU's), there remains a small number of persistent organisms which are detectable by, for example, broth dilution counting. These resistant subpopulations are phenotypically resistant to rifampicin, since they become sensitive to rifampicin at normal MIC levels upon resumption of growth.

Analogous studies using stationary phase *E. coli* and *S. aureus* model systems and treatment thereof with kanamycin and ampicillin respectively have yielded similar results.

Such phenotypically resistant subpopulations of stationary phase bacteria, obtainable by treating stationary phase bacteria with a high dosage of an antibacterial agent, constitute one feature of the present invention.

The nature of the antibacterial agent employed may depend on the particular bacteria being investigated. The use of antibacterial agents such as rifampicin which target RNA polymerase, for example at concentrations of 10^2, 10^3 or 10^4 times the normal MIC level, has been found convenient. In a representative embodiment of this aspect of the invention, treatment of stationary phase *M. tuberculosis* at a level of 10^6 bacteria/ml for one day with 100 μg/ml of rifampicin (MIC x 10^3) resulted in a phenotypically resistant subpopulation of 10^2 bacteria/ml. In further embodiments, treatment of stationary phase *E. coli* or *S. aureus* ($\tilde{=}$ 10^9 bacteria/ml) with 50 μg/ml kanamycin or 100 μg/ml ampicillin for 3 days results in a phenotypically resistant subpopulation of $\tilde{=}$ 10^5 bacteria/ml.

On the basis that the phenotypically resistant subpopulations of the invention mimic the behaviour of dormant bacteria *in vivo*, they constitute valuable tools in screening procedures designed to identify potentially valuable new antibacterial agents. It will be

appreciated that antibacterial agents having activity against such phenotypically resistant subpopulations may have great therapeutic potential in the treatment of diseases such as tuberculosis in which dormant bacteria provide a pool for reinfection.

Such processes, for example comprising the steps of:

(i) growing a bacterial culture to stationary phase;

(ii) treating said stationary phase culture with an antibacterial agent at a concentration and for a time sufficient to kill growing bacteria, thereby selecting a phenotypically resistant subpopulation;

(iii) incubating a sample of said phenotypically resistant subpopulation with test compounds; and

(iv) assessing any antibacterial effects against said phenotypically resistant subpopulation, constitute a further feature of the invention.

Such a process facilitates the rapid screening of large numbers of compounds quickly and efficiently. The compounds to be screened may be any chemical compounds and may be already known or may themselves be novel. Especially suitable candidate compounds are the products of combinatorial chemistry, phage display libraries, natural products, synthetic, semi-synthetic or natural homologues, analogues, derivatives and precursors of known antimicrobial agents or other pharmaceuticals. Candidate compounds identified in such a manner, for example exhibiting a minimum inhibitory concentration of 1 μg/ml or less in respect of the resistant subpopulation, may then be subjected to further analysis, efficacy and toxicity tests etc.

In a further aspect, the present invention is directed towards novel chemical compounds which exhibit bacteriostatic or bactericidal effects on an antibacterial agent resistant subpopulation of stationary phase bacteria, e.g. a rifampicin-resistant

subpopulation of *M. tuberculosis*, a kanamycin-resistant subpopulation of *E. coli* or an ampicillin-resistant subpopulation of *S. aureus*.

In another aspect, the present invention is directed towards a composition or formulation comprising a novel antimicrobial agent identified by the process described herein and a pharmaceutically acceptable diluent or excipient.

In the clinical management of tuberculosis and other disease states where the establishment and existence of dormant bacteria is problematic, it may be advantageous to administer a combination of antibacterial agents which are respectively directed towards the different growth phases of these organisms. Thus, for example, an appropriate chemotherapeutic approach to the treatment of tuberculosis would be to administer to a patient one or more antibacterial agents directed against the growing or log-phase of the microorganisms and one or more antibacterial agents directed against the dormant or stationary phase population.

Thus, a preferred formulation according to the present invention comprises at least one antibacterial agent which has activity against actively growing bacteria and at least one antibacterial agent having activity against the phenotypically resistant subpopulation of the stationary phase of said bacteria. Such formulations may be presented as a combined preparation for simultaneous, separate or sequential use in the treatment of bacterial infections such as tuberculosis.

In a further aspect, the present invention provides the compounds identified as effective against phenotypically resistant subpopulations of stationary phase bacteria by the above screening process for use in the treatment of bacterial infection.

In yet another aspect, the present invention

relates to the use of compounds identified as effective against phenotypically resistant subpopulations of stationary phase bacteria by the above screening process in the preparation of a medicament for the treatment of bacterial infection.

The use of such antibacterial compounds identified by the above process in the treatment of bacterial infections involving dormant bacteria constitutes another aspect the present invention.

Alternatively viewed, the present invention provides a method of treatment of a bacterial infection comprising administering to a patient in need of such therapy an effective amount of an antibacterial agent directed towards the stationary phase of growth, optionally in the presence of one or more antibacterial agents directed towards the growing phase of said organism.

In each aspect of the invention, the stationary phase bacteria which are the target of the the antibacterial agents may include pathogenic bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Streptoccus pyogenes*, *Streptococcus gordonii*, *Eschericia coli* and particularly preferably, *Mycobacterium tuberculosis*.

The following non-limitative Examples serve to illustrate the invention. In the accompanying drawings:

Fig. 1 shows growth curves for *E. coli* and *S. aureus* over a period of 10 days;

Figure 2 shows the viability of *E. coli* after treatment with 50 μg/ml kanamycin during stationary phase and log phase growth;

Figure 3 shows the viability of *S. aureus* after treatment with 100 μg/ml ampicillin during stationary phase and log phase growth.

EXAMPLE 1

Growth of *M. tuberculosis* and selection for a phenotypically but not genetically rifampicin resistant population of cells

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth containing 0.05% Tween 80 supplemented with 10% ADC, without agitation or other disturbance for up to 100 days, in accordance with the procedure of Wayne (1976) Amer. Rev. Resp. Dis. 114: 807-811. Where the stationary phase organisms were viable they were resistant to MIC levels of 0.1 μg/ml rifampicin.

Sixty 10 ml samples of the cultures were vortexed with glass beads for 3-5 minutes, followed by sonication in a water bath sonicator for less than 5 minutes. All the cultures were pooled into a 500 ml sterile bottle with screw cap.

Prior to addition of rifampicin, samples of the culture were taken to check purity, viability and metabolic activity as follows:

1. 2 x 100 μl for CFU counts
2. 2 x 5 ml for broth counts
3. 2 x 10 ml for [^{3}H]uridine counts
4. 2 x 5 ml for [^{35}S]methionine counts
5. 10 ml for RNA extraction for RT-PCR
6. 10 ml for drug-free control
7. 20 μl for blood agar to check sterility

Selection and detection of a rifampicin-resistant subpopulation

50 ml of 1000 μg/ml rifampicin was added to 500 ml of the above culture to obtain a final rifampicin

concentration of 100 μg/ml, and the culture was incubated at 37°C for 5 days. The cells were then harvested in sterile 50 ml tubes by centrifugation at 5000g for 15 minutes, then washed twice in sterile PBS containing 0.05% Tween 80 and Selectitab antibiotics (which comprises a combination of antibacterial agents which kills most bacteria but not *M. tuberculosis*, thus preventing *M. tuberculosis* from becoming overgrown by faster growing bacteria). The cells were resuspended in 500 ml fresh 7H9 medium.

Sterility of the culture was checked before further experiments. 2 x 100 μl samples of the culture was added to blood agar plates in duplicate which were incubated at 37°C overnight. The cell suspension was kept at 4°C overnight. Contaminated cultures should always be discarded.

It was found that this treatment with high levels of rifampicin reduced plate counts to zero colony forming units but resulted in a small number of persisting organisms which were detectable by broth dilution counting. Table 1 shows a typical example of such selection where 10^2 bacteria/ml remained from 10^6 after one day of rifampicin treatment. From this, it appears that *M. tuberculosis* has at least two populations of stationary phase organisms: the first is killed by high dose rifampicin and the second 'persists' and is phenotypically resistant. Upon resumption of growth, the rifampicin-resistant stationary phase subpopulation becomes sensitive to rifampicin at the normal MIC level of 0.1 μg/ml.

EXAMPLE 2

Characterisation of the rifampicin-resistant subpopulation

Samples of the rifampicin resistant subpopulation of cells were analysed and the cell population further characterised. The population was shown to be metabolically active by virtue of transcriptional and translational activity detected in the population.

The cells were shown to be very responsive to changes in their environment. When rifampicin was removed and replaced by growth medium alone, the level of transcription of four genes analysed increased by 5- to 10-fold after 12 hours. Radioactive uridine incorporation also increased about 5-fold after removal of rifampicin and incubation with medium alone (Table 1 - compare counts at 5* and 5 days). This suggested that the level of transcription increased rapidly under these circumstances. The data do not exclude the possibility that the organisms replicate in the presence of fresh medium, although this would only account for a two-fold rise in transcription in 12 hours, since the generation time of *M. tuberculosis* is about 20 hours. The data support a hypothesis that, in this *in vitro* model of drug resistant stationary-phase bacteria, there is a major population of bacteria that are actively transcribing RNA and are environmentally reactive, yet remain plate culture negative.

To distinguish between phenotypic and genotypic resistance the bacteria thoroughly washed after rifampicin treatment and cultured in liquid 7H9 medium for 6 weeks. In four separate experiments *M. tuberculosis* was invariably grown. These bacteria were sensitive to 0.1 μg/ml rifampicin and were negative for

rifampicin resistant mutations in rpoB as detected by RT-PCR and hybridisation with oligonucleotide probes (Immunogenetics N.V., Netherlands; data not shown). This indicates that the resistance is phenotypic in this model.

It is unlikely that the mechanism of induced resistance depends on mutation or reduced levels of expression of rpoB mRNA (i.e. resulting in a reduced level of drug target) because transcription continues even in the presence of rifampicin (Table 1).

Table 1

Incorporation of [^{3}H]-uridine into *M. tuberculosis* after addition of rifampicin

Days in rifampicin	Plate coutns	Broth counts	[^{3}H]-uridine
0	6.6×10^5	10^6	74682±630
1	0	10^2	2228±88
2	0	10^2	2316±120
3	0	10^2	2430±54
4	0	10^2	2318±126
5	0	10^2	2388±20
5*	0	10^2	518±10
0	0 (heat-killed)	0	180±19

Legend

M. *tuberculosis* was grown in 7H9 medium containing 0.05% of Tween 80 supplemented with 10% ADS (Difco Laboratories) without shaking for 100 days. Rifampicin was added to the cultures at a final concentration of 100 µg/ml for 5 days. Viability was estimated at one day intervals. The cells were thoroughly washed and 100 µl of samples from 10-fold dilutions of the cultures

were added to triplicate plates of Middlebrook 7H11 medium supplemented with OADC (Difco Laboratories). Colony forming units (CFU's) were counted after incubation of the plate for 3 weeks at 37°C. Broth counts were performed at 10-fold dilutions by adding 1 ml of the sample to 9 ml of 7H9 medium. Viability was estimated by examining growth in the diluted cultures after incubation for 6 weeks at 37°C.

For incorporation of radioactive uridine, 10 ml of the culture for each time point was washed twice to remove the remaining rifampicin and then resuspended in 10 ml 7H9 medium followed by incubation with 10 μCi/ml of [^{3}H]-uridine for 20 hours. For 5* the culture was not washed after incubation with rifampicin and 10 μCi/ml of [^{3}H]-uridine was added to the culture which was further incubated in the presence of rifampicin for 20 hours. The RNA was extracted by methods known in the art. The RNA [^{3}H]-uridine incorporation was determined as counts per minute of trichloroacetic acid precipitated RNA. The results were confirmed in three independent experiments.

Susceptibility assessment of drug libraries

Sterile 0.7 ml labelled transparent plastic snap-capped tubes containing 1-20 μg of the compound to be tested are used. The compound is dissolved in 0.25 ml of sterile distilled water or other appropriate diluent. 0.25 ml of the rifampicin treated culture is added to each tube in the class I cabinet in a category III safety containment laboratory with care being taken to avoid contamination. The tubes are incubated at 37°C. The drug effects are examined by CFU counts by addition of 2 X 50 μl of each sample to 7H11 agar plates in duplicate including 2 drug-free controls at 1 week intervals. A series of 10-fold dilutions of the samples may be required, which is made in 7H9 broth with 0.05%

Tween 80 but without ADC, then the diluted samples are plated on 7H11 agars and the concentration effect of the candidate compounds determined.

EXAMPLE 3

Selection of phenotyically but not genotypically resistant subpopulations of *E. coli* and *S. aureus* using kanamycin and ampicillin respectively

Growth of *Escherichia coli* and *Staphylococcus aureus*

Escherichia coli K12 and *Staphylococcus aureus* are grown in 10 ml of nutrient broth No. 2 (Oxoid) with continuous shaking at 120 rpm for 10 days. Viability of the bacteria is estimated by colony forming unit counts at 2 hours intervals for the first 24 hours and 12-24 hours afterward. From serial 10-fold dilutions of the experimental cultures, 100 µl samples are added to triplicate plates of nutrient agar plates (Oxoid). Colony forming units (CFU) are counted after incubation of the plates at 37°C for 24 hours.

Selection of persistent bacteria by antibiotics

Ampicillin and kanamycin are added to 5-day stationary-phase cultures of *E. coli* and *S. aureus* respectively to a final concentration of 100 µg/ml and 50 µg/ml respectively for 3 days. After 3 days of antibiotic treatment, the cells are washed with sterile distilled water 3 times, then resuspended in 10 ml fresh nutrient broth. Viability is estimated by CFU counts and broth dilution counts. Broth dilution counts are performed in a serial 10-fold dilution in nutrient broth, then 1 ml of each dilution in added into 9 ml of nutrient broth in 30-ml universal tubes in triplicate. The growth curves for *E. coli* and *S. aureus* over the 240 hour period in

the absence of antibiotics is shown in Fig. 1. The effect of antibiotic exposure on the *E. coli* and *S. aureus* during both log and stationary phase of growth is shown in Figs. 2 and 3. Treatment of the cultures during stationary phase results in the selection of an antibiotic resistant subpopulation of cells.

Claims

1. A phenotypically antibiotic-resistant subpopulation of stationary phase bacteria, obtainable by treating stationary phase bacteria with a high dosage of an antibacterial agent.

2. A phenotypically antibiotic-resistant subpopulation of stationary phase bacteria as claimed in claim 1 wherein said bacteria are *Staphylococcus aureus*, *Eschericia coli*, *Haemophilus influenzae*, *Streptoccus pyogenes*, *Streptococcus gordonii* or *Mycobacterium tuberculosis*.

3. A process for screening for agents having antibacterial activity against stationary phase bacteria comprising the steps of:

(i) growing a bacterial culture to stationary phase;

(ii) treating said stationary phase culture with an antibacterial agent at a concentration and for a time sufficient to kill growing bacteria, thereby selecting a phenotypically resistant subpopulation;

(iii) incubating a sample of said phenotypically resistant subpopulation with test compounds; and

(iv) assessing any antibacterial effects against said phenotypically resistant subpopulation.

4. A process as claimed in claim 3 wherein said bacteria are *Mycobacterium tuberculosis* and said antibacterial agent is rifampicin.

5. A process as claimed in claim 3 wherein said bacteria are *Eschericia coli* and said antibacterial agent is kanamycin.

6. A process as claimed in claim 3 wherein said

bacteria are *Staphylococcus aureus* and said antibacterial agent is ampicllin.

7. Chemical compounds which exhibit antibacterial activity against a phenotypically antibiotic resistant subpopulation of bacteria as defined in claim 1 or claim 2.

8. A composition comprising an antibacterial agent as defined in claim 7 and a pharmaceutically acceptable excipient or diluent.

9. A formulation comprising at least one antibacterial agent having activity against actively growing bacteria and at least one antibacterial agent having activity against a phenotypically antibiotic-resistant subpopulation of stationary phase bacteria wherein said formulation is presented as a combined preparation for simultaneous, separate or sequential use in the treatment of bacterial infections.

10. The use of antibacterial compounds as defined in claim 7 in the treatment of bacterial infections involving dormant bacteria.

11. Use of antibacterial compounds as defined in claim 7 in the preparation of a medicament for the treatment of bacterial infections involving dormant bacteria.

12. A method of treating of a bacterial infection comprising administering to a patient in need of such therapy an effective amount of an antibacterial compound as defined in claim 7.

13. A method as claimed in claim 12 further comprising administration of one or more antibacterial agents directed towards growing bacteria.

Growth curves of *Escherichia coli* and *Staphylococcus aureus*

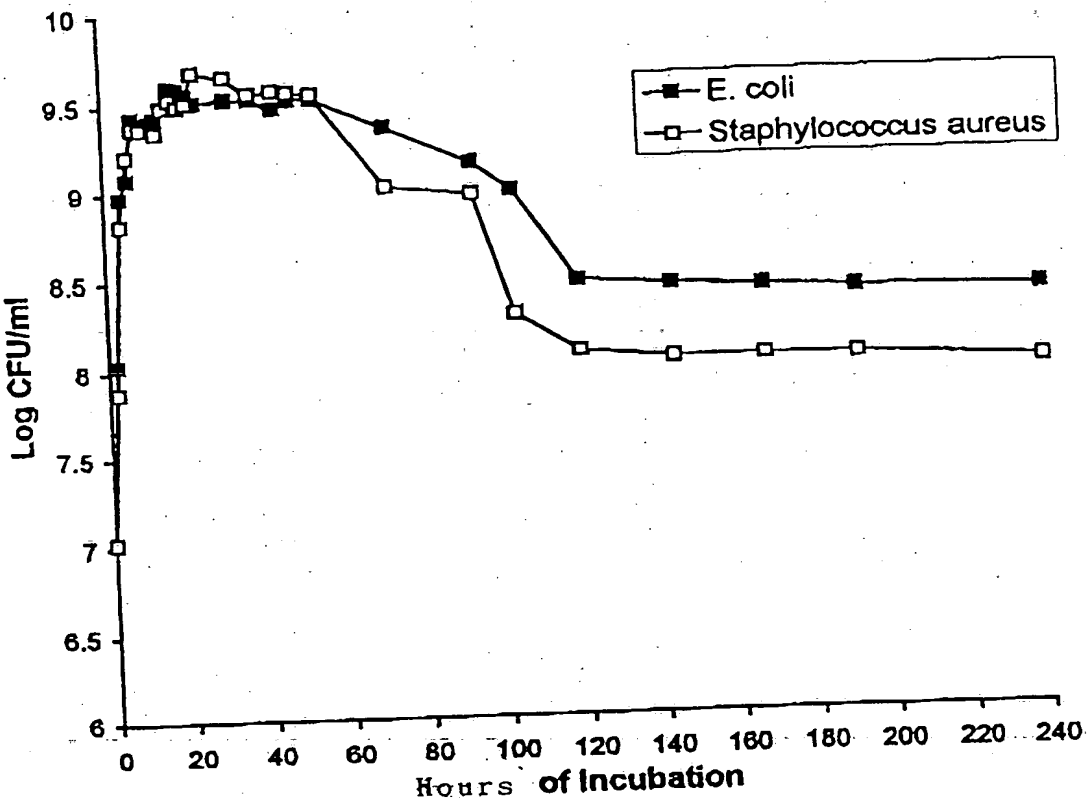

Fig. 1

Viability of *Escherichia coli* after Treatment with Antibiotics

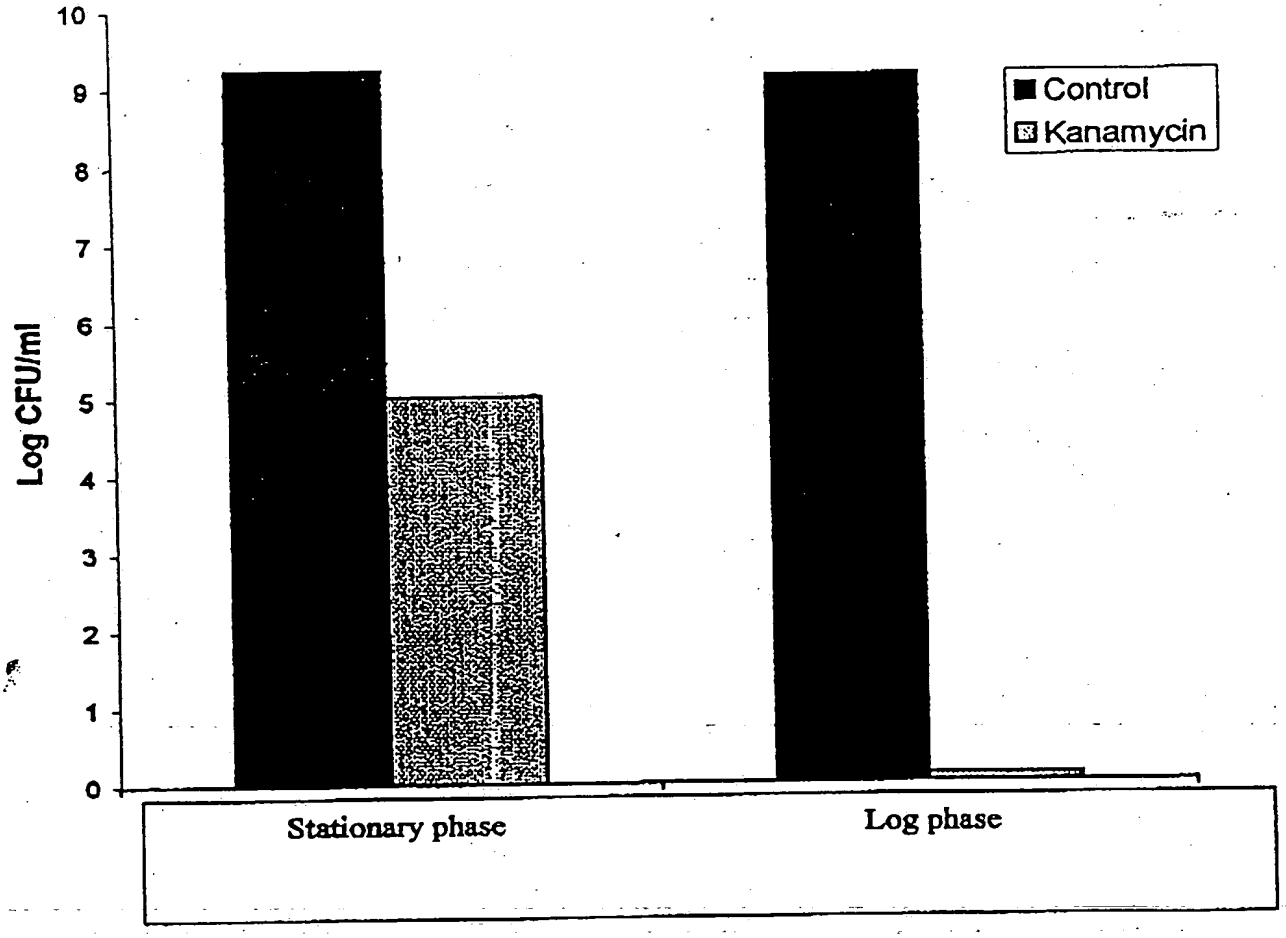

Fig. 2

Viability of *Staphylococcus aureus* after Treatment with Antibiotics

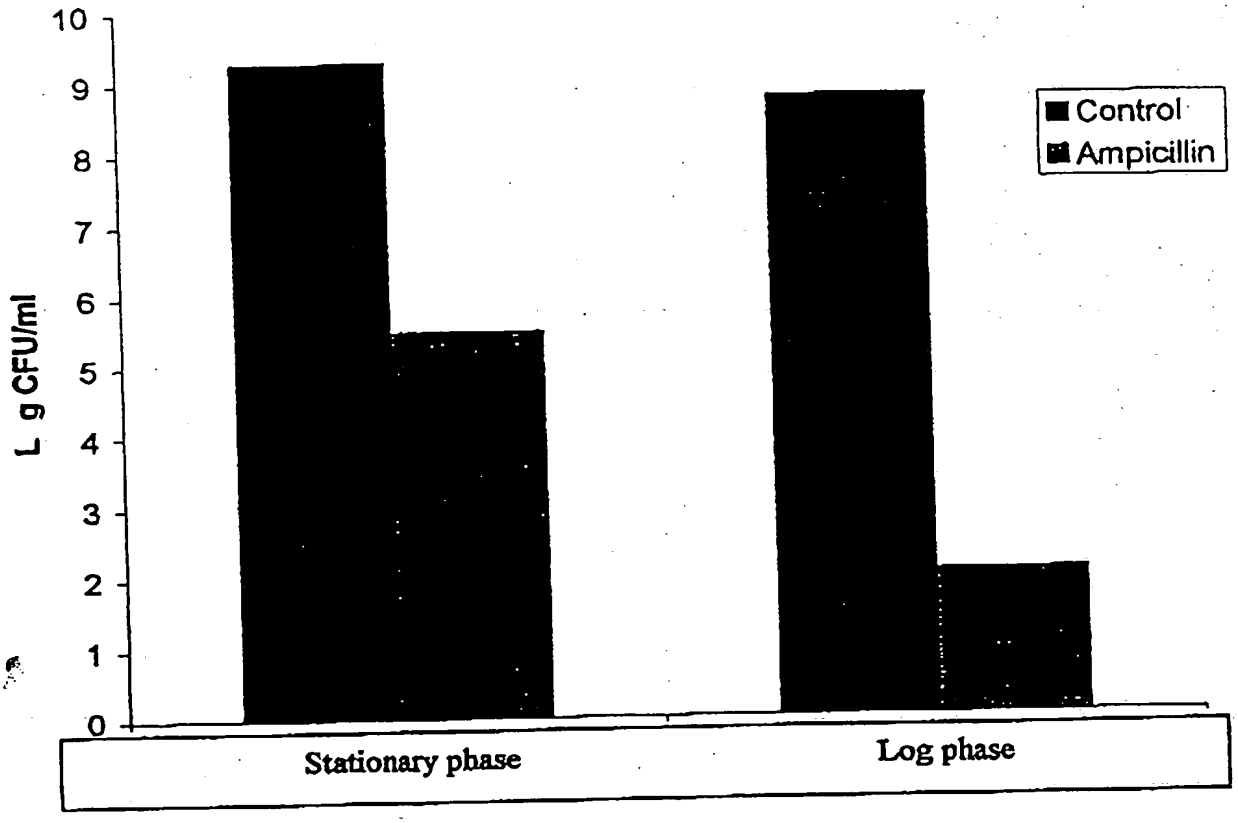

Fig. 3